Development 113, 1-26 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

Spatial mechanisms of gene regulation in metazoan embryos

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Summary

The basic characteristics of embryonic process throughout Metazoa are considered with focus on those aspects that provide insight into how cell specification occurs in the initial stages of development. There appear to be three major *types* of embryogenesis: *Type 1*, a general form characteristic of most invertebrate taxa of today, in which lineage plays an important role in the spatial organization of the early embryo, and cell specification occurs *in situ*, by both autonomous and conditional mechanisms; *Type 2*, the vertebrate form of embryogenesis, which proceeds by mechanisms that are essentially independent of cell lineage, in which diffusible morphogens and extensive early cell migration are particularly important; *Type 3*, the form exemplified by long germ band insects in which several different regulatory mechanisms are used to generate precise patterns of nuclear gene expression prior to cellularization. Evolutionary implications of the phylogenetic distribution of these types of embryogenesis are considered. Regionally expressed homeodomain regulators are utilized in all three types of embryo, in similar ways in later and postembryonic development, but in different ways in early embryonic development. A specific downstream molecular function for this class of regulator is proposed, based on evidence obtained in vertebrate systems. This provides a route by which to approach the comparative regulatory strategies underlying the three major types of embryogenesis.

Key words: gene regulation, spatial mechanism, metazoa, cell lineage, cell specification.

Introduction

As we learn how embryos work, we begin to see the relations between their external cellular and morphological processes and the underlying regulatory mechanisms. How embryos of diverse forms utilize their cell lineage, how they position differentially functioning cells with respect to their three-dimensional body plans, and how and when they initially carry out cell specification, reveal functions that must be served by their developmental gene regulatory systems. The primary role of the regulatory systems that drive the initial construction of the embryo is of course to establish differential patterns of gene expression in cells located in different regions. However, an embryo is not simply equivalent to a set of differentiating cells, even a spatially organized set. A particular function of embryonic cells is *to interact* in specific ways, in order to generate morphological structure. The nature of the developmental process provides information both about how an embryo arranges the presentation of different sets of active transcriptional regulators in its different cells, and about the location and role of specific intercellular ligand–receptor interactions, that in turn will further affect the internal regulatory milieu of these cells.

While the relation between external embryonic character and underlying regulatory process is in principle evident, it is also intellectually disturbing, because metazoan embryos differ so greatly in their external character. *Caenorhabditis elegans* embryos have an invariant cell lineage while the cell lineage of a chicken or mouse or fish embryo is always different from that of another of the same species; portions of sea urchin or jellyfish embryos can regulate to generate whole new embryos, while equivalent portions of ascidian or annelid embryos cannot; *Drosophila* embryos specify elegant spatial patterns of gene expression before there are any cells to interact, while in *Xenopus* or sea urchin embryos the initial spatial diversification of gene expression depends causally and extensively on intercellular interaction. The two fundamental issues that I shall try to address here are: what are the common regulatory organizations that underlie the diversity of observed processes in metazoan embryos, and how might these organizations have arisen in evolution; that is, is there a simple phylogenetic sense that one might make out of their comparative distribution. These are not necessarily intertwined issues, but I shall deal with them as such. As our predecessors in embryology were well aware a century ago, comparative considerations can be immensely illuminating, and, even at the advanced state to which we have been carried by our superb molecular technology, it might still be said that we need all the help we can get in trying to understand embryonic development.

I shall approach these problems by the following route. There appears to be an associated set of characters that define a canonical type of embryonic process found in many, though not all, invertebrate groups. One can reasonably infer, on present knowledge, the outlines of the molecular modus operandi of these embryos. However, I shall argue that their regulatory organization is clearly not directly applicable either to advanced insect or to vertebrate embryos, nor are most of the mechanisms by which insect and vertebrate embryos initially organize themselves directly applicable to one another. The phylogenetic occurrence of these three embryo types leads to the strong conclusion that advanced insect and vertebrate embryogenesis are derived processes that differ, amongst other things, in their initial use of homeodomain regulators (i.e. the DNA-binding regulatory protein products of homeobox genes). I suggest a specific downstream developmental function for homeodomain regulators, and this can be utilized to explore further the general mechanisms that in each of these three basic forms direct the transformation of egg into organism.

The canonical invertebrate embryo

Those characters that are most useful and most important to consider reveal the processes of cell specification as embryogenesis begins, i.e. the processes by which are initially established the identity, and the fates, of the progeny of individual blastomeres. An analysis of such characters in a recent comparative review (Davidson, 1990) showed that similar regulatory strategies apparently underlie embryogenesis in four entirely unrelated organisms, *viz* the nematode *Caenorhabditis*, the sea urchin *Strongylocentrotus*, the neogastropod mollusc *Ilyanassa*, and the ascidian *Ciona*. By most criteria this is of course an impossibly diverse group of organisms, and of embryonic forms. Nematodes are typically direct developing terrestrial animals of distant protostome affinity, that lack a true (i.e. mesoderm-lined) coelom, and the embryos of which display a unique non-spiral form of cleavage; while neogastropods such as *Ilyanassa* are advanced marine coelomate protostomes that utilize classic spiral cleavage in embryogenesis and give rise to a special form of free-living larva. The ascidian *Ciona* and the sea urchin *Strongylocentrotus* are both indirectly developing lower deuterostomes, but in traditional classifications the embryos of these groups present an archetypical contrast: sea urchin embryos are renowned for their amazing regulative abilities, while ascidian embryos are famous for the opposite quality. Yet, if the focus is set upon certain essential features of early development, there emerges a distinctive suite of key developmental characters that is shared by all four of these disparate forms, as follows. (i) Within each species, cleavage is more or less invariant, meaning that the cleavage planes are in approximately the same positions in each individual relative to the axes of the egg, and in the undisturbed embryo the lineages descended from specific blastomeres display generally predictable, invariant fates. (ii) One axis of the egg is preformed, while the second is set up after fertilization, by processes that are various and specific to each phyletic group (the mechanism of second axis formation is thus not a general or fundamental character). (iii) Where studied with molecular markers, histotypic specification and the appearance of an initial set of differentially functioning cell types is found to occur before gastrulation in all regions of the embryo, and to *precede* any large-scale embryonic cell migration (though when cells do become migratory they may add to or change their differentiated properties further). (iv) Some founder cells of the lineages of which the early embryo is composed are specified *autonomously*, while others, within the context of the invariant lineage, are specified *conditionally*. Since this last character leads us directly to the underlying embryonic processes, it is important that at the outset of this discussion we explore the implications of these fundamental modes of initial blastomere specification.

Modes of blastomere specification, and the basis of regulative development

Automonous specification signifies a process in which the identity of a lineage founder cell depends only on its internal constituents, and is independent of contact with other coexistent cells. Thus if a blastomere inherits maternal factors that are necessary and sufficient for its specification, it will exhibit the same functions irrespective of what other cells are next to it. If isolated, its progeny will do so even in culture, given that conditions are acceptable. In contrast, the identity of a *conditionally specified* founder cell depends at least in part on local intercellular interactions. The state of differentiation displayed by lineages descendant from a conditionally specified founder blastomere thus depends on the location in the embryo of this blastomere. In embryos with invariant lineage, such interactions are generally short range (i.e. compared to blastomere diameter). Thus changing the immediate neighbors of a prospective founder blastomere can result in a change in its own identity, as it may receive different signals from the blastomeres to which it is now apposed than it would have in its original position. To the extent that an embryo can *select* among a number of different potential cell fates by specific intercellular interactions, it does not have to specify all of these fates autonomously, but to position pluripotential cells in the right place that can give the right response. Thus, *ab initio*, founder cells that are to be specified conditionally possess more potentialities than they will display in any given embryo, normal or chimeric, while, on the other hand, autonomously specified founder cells have no functional need for embryonic plasticity. There are two corollaries that are important for what follows. First, in embryos with invariant cell lineage, founder cell specification, including conditional specification, always begins during cleavage, and it follows that the selective intercellular interactions also must

occur during cleavage. Second, because conditionally specified blastomeres are initially pluripotential, they can generate what embryologists term *regulative development*, as perceived in ectopic or chimeric blastomere recombinations, or in partial embryos. In these situations, blastomeres normally subject to conditional specification can give rise to cell types that would be missing were the lineages normally generating these cell types their sole possible source (see Davidson, 1989, 1990 for discussion and examples). The observation of regulative behavior reliably indicates a conditional specification process. This is useful, since it is unfortunately often the case that no detailed experimental examination of blastomere potentialities has been carried out on embryos belonging to many of the less well studied groups.

Autonomous specification and lineage specific gene expression

We now consider briefly how embryos that display all of the external characters indicated above are likely to work, with illustrative examples drawn in each case from *several* of the four embryos so far mentioned. For convenience in the following I shall refer to embryos that display these characters as *Type 1* embryos. In Type 1 embryos the means by which the primordial cytoarchitectural polarity of the egg is transmuted into an axial feature of the body plan of the embryo, is the autonomous specification of lineage founder cells *at (at least) one pole of the original egg axis*. All four of our initial Type 1 embryos provide examples: in *C. elegans*, in which the A/P axis is primordial, the autonomously specified germline (P) and body wall muscle (D, Cap and Cpp) lineages arise at or near the posterior end of the egg (Strome, 1989; Wood, 1991; reviewed in Davidson, 1986, 198–212, 436–445); in ascidians the endodermal lineages (A6.1, A6.3, B6.1 founder cells) arise at the vegetal pole of the primordial D/V (=An/Vg) axis (Jeffery, 1990; Whittaker, 1990); in molluscan eggs endoderm lineages also arise at the vegetal pole of the primordial An/Vg axis; and in sea urchin embryos the autonomously specified skeletogenic founder cells (VAMk, VOMk and VLMk cells) arise at the vegetal pole of the primordial An/Vg axis. Autonomously specified founder cells may also be formed *at (at least) one pole of the second axis of the egg*. In ascidian eggs, for example, one of the most intensely studied cases of an autonomous embryonic cell lineage is that giving rise to the larval tail muscles, the founder cells for which arise within the lineages of the bilateral B4.1 cells formed at the posterior pole of the second, or A/P axis of the embryo. Extensive evidence (Meedel *et al.* 1987; reviewed in Davidson, 1986, 430–436, 489–491; Davidson, 1990) indicates that specification of the founder cells depends on maternal factors, localized in the particular region of egg cytoplasm that is inherited by the B4.1 cells. The localization of these factors – with respect to the invariant cleavage planes – occurs through a cytoskeletal cortical reorganization of the egg, following fertilization (reviewed by Davidson, 1986, 489–493; Bates and Jeffery, 1988; Jeffery, 1990). The *Ilyanassa* embryo provides a second example of an autonomous lineage arising at a pole of the second axis, *viz* the specification by maternal factors of the mesoderm germ band founder cells, as these factors are sequestered into the D macromere and then certain of its progeny (reviewed in Davidson, 1986, 453–473).

Studies with molecular markers demonstrate the early expression of cell-type-specific genes in the autonomous lineages of *C. elegans* and sea urchin embryos. For example in *C. elegans* the terminal muscle differentiation regulator MyoD begins to be expressed at mid-cleavage in the immediate daughters of the body wall muscle founder cells, i.e. within one cell cycle following the segregation of the D, Cap and Cpp cells from sister cells of other lineages (Krause *et al.* 1990). In sea urchin (*S. purpuratus*) embryos, mRNA for SM50, a skeletal matrix protein, begins to accumulate within 2–3 cleavage cycles after completion of segregation of the skeletogenic founder cells at 5th cleavage (Benson *et al.* 1986; Killian and Wilt, 1989). Furthermore, as might be expected if autonomously specified blastomeres contain localized regulatory factors that control the activation of their lineage-specific genes, exogenous fusion genes under the control of the relevant marker gene regulatory sequences are activated appropriately in autonomous lineages. This was demonstrated with a *MyoD–lacZ* gene fusion in *C. elegans* (Krause *et al.* 1990), and with an *SM50–CAT* gene fusion in *S. purpuratus* (Sucov *et al.* 1988). Gene regulatory factors that are sequestered into the cytoplasmic domains of autonomous founder cells would be divided up amongst the clonal descendants of their lineages as cleavage progresses. As I discussed earlier (Davidson, 1990), cytochalasin experiments that have been carried out on ascidian and nematode eggs support this mechanism of autonomous founder cell specification. These experiments show in essence that at whatever cleavage stage cytokinesis is blocked by cytochalasin treatment, those blastomeres that then contain cytoplasmic regulatory factors destined for autonomous lineages ultimately express their lineage-specific markers (for *C. elegans*, see Cowan and McIntosh, 1985; Edgar and McGhee, 1986; for ascidians, Whittaker, 1973, 1980, 1990; Crowther and Whittaker, 1986; Meedel *et al.* 1987; Nishikata *et al.* 1988; Jeffery, 1989). That is the regulatory factors function in whatever nuclei are included within the domains bounded by the cell membranes present when the drug was added. Thus in the *undisturbed Type 1 embryo, the correct positional location of these gene regulatory domains depends directly on the location of the cleavage planes bounding the autonomously specified founder cells, with respect to the axes of the egg*.

Conditional specification and the spatial regulation of gene expression in Type 1 embryos

Founder cells of Type 1 embryos that are specified conditionally may constitute a minor fraction of the embryo, as in ascidian embryos, or far more commonly, a large fraction as in *C. elegans* or sea urchin embryos. Lineages of the ascidian embryo that *are* conditionally

specified include those responsible for brain, sensory pigment cells and terminal caudal tail muscle formation (see, e.g. Meedel *et al.* 1987; Nishida and Satoh, 1989; other references cited in Davidson, 1990). In *C. elegans* probably the entire set of anterior (AB) and likely other lineages as well are conditionally specified (it is revealing that in contrast to the autonomous D lineage discussed above, the AB and a large part of the MS lineages are not clonal, in that entirely different cell types arise from sister cells throughout cleavage; Sulston *et al.* 1983). In sea urchin embryos all of the oral (including neurogenic), and aboral ectoderm lineages, and probably elements of the gut lineages as well are conditionally specified. In ectopic recombinations virtually all embryonic cell fates can be elicited from pluripotential cells that in the unfertilized embryo normally undergo conditional specification (Hörstadius, 1939, 1973; for interpretation see Davidson, 1986, 505–509; Wilt, 1987; Davidson, 1989). Since conditional specification implies regulative ability, and *vice versa*, differences in the extent of conditional specification account for the large differences amongst embryos in their regulative capacity. For example, ascidian embryos are unable to compensate for missing or killed cleavage stage blastomeres, or to regenerate missing right or left halves (Conklin, 1905; Reverberi *et al.* 1960; Ortolani, 1987), whereas *C. elegans* embryos can respecify their asymmetric right/left lineage fates if the positions of the descendants of the AB blastomere are shifted experimentally early in cleavage (Wood, 1991). Similarly, exchange of the positions of certain anterior blastomeres of *C. elegans* causes reversal of the second (D/V) axis (Priess and Thomson, 1987).

Like autonomously specified lineages in Type 1 embryos, conditionally specified lineages transcriptionally activate particular cell-type-specific marker genes very early in development. Examples include aboral ectoderm markers in the sea urchin embryo such as CyIIIa cytoskeletal actin, or the *Spec*I Ca^{2+}-binding protein, the mRNAs for which appear before cleavage is complete (Lynn *et al.* 1983; Shott *et al.* 1984; Lee, 1986; Lee *et al.* 1986; Hickey *et al.* 1987). Ascidian embryos produce tyrosinase, a marker of the conditionally specified pigment cell lineage, within a few hours of the end of cleavage, at about the same time as markers of autonomously specified lineages appear (Nishida and Satoh, 1989; development in *Halocynthia roretzi*, as in other ascidians, is so rapid that this is already neural plate stage). The first point is thus that conditional specification in Type 1 embryos results in immediate activation of downstream batteries of histotypic genes, in blastomere lineages that remain located *in situ* in more or less the same positions in which their founder cells were born (just as in autonomously specified lineages). Immediate conditional activation of such lineage markers has been studied most extensively in cultured combinations of blastomeres in sea urchin embryos. Thus for example blastomeres from the animal pole tiers, that in undisturbed embryos produce only oral and aboral ectodermal derivatives, are made to express a gut alkaline phosphatase marker if combined with skeletogenic micromeres (Khaner and Wilt, 1990). The same result, and also the expression of SM50 mRNA, can be elicited by treatment of animal blastomeres with Li^+ (Livingston and Wilt, 1989).

A second point is that the conditional specification functions monitored by these markers may be mediated by known signal transduction pathways such as the phosphatidyl inositide cycle, and/or tyrosine kinase-linked signal receptors. A role for the phosphatidyl inositol pathway is probably required to explain the frequently observed developmental effects of Li^+ (Berridge *et al.* 1989). Signal transduction effects on gene regulatory systems, mediated by specific ligand–receptor interactions, provide an obvious and general starting point in considering conditional specification in embryos. Recently many examples of molecules belonging to known families of ligands that activate signal transduction systems *via* specific receptors have been identified in amphibian, mammalian and *Drosophila* embryos. For Type 1 embryos, a good example is provided by the *glp-1* locus of *C. elegans* (Austin and Kimble, 1989). This gene, the product of which encodes a receptor with EGF-like repeats, is required for the conditional specification of pharyngeal muscle cells descendant from the AB blastomere (Priess *et al.* 1987), but not for the apparently autonomously specified pharyngeal muscle cells descendant from the MS blastomere. In addition, though there are yet no functional data, mRNAs encoding apparent ligands of the EGF family have been cloned from very early sea urchin embryos (Delgadillo-Reynoso *et al.* 1989; Grimwade *et al.* 1991) as has a member of the TGFβ family that resembles *Drosophila dpp* (J. L. Micol and E. H. Davidson, unpublished).

Since it is so essential and widespread an aspect of early Type 1 development, one of our most important objectives must be to erect a working hypothesis that provides a general and testable molecular model for conditional specification, and I recently suggested a possible mechanism (Davidson, 1989, 1990). Given a range of receptors, the effect of a particular ligand interaction in the early Type 1 embryo can be accounted for as follows (Davidson, 1989, 1990): prior to conditional specification blastomeres can be envisioned to contain key maternal gene regulatory factors (or their maternal mRNAs) but these factors initially exist in a cryptic or inactive state. *Selection* of the appropriate gene expression program would thus depend on which set of such factors a specific inductive signal transduction will affect, i.e. by activating them by means of covalent modification, mobilization of their access to the nucleus from a site of sequestration in the cytoplasm, or the presentation of an active cofactor. The initial degree of pluripotentiality of such a blastomere might be defined directly by the range of such factors or factor sets that it contains. The selection of the particular pattern of differential gene expression would then depend on the particular intercellular interaction in which a blastomere at a given position in the canonical cleavage pattern engages (this of course

includes interactions with regulatory factors that function negatively, in that they repress gene expression, as well as positive interactions). Were a blastomere moved to another position it might encounter a different ligand, presented by a different neighboring blastomere, and it would be specified differently, which we might notice as a remarkable example of regulative behavior.

The organization of the initial body plan in Type 1 embryos may thus begin with the presentation of particular ligands on the axially positioned, *autonomously* specified founder cells. These will affect the conditional specification of the adjacent blastomeres, thus selecting their identity. Once again, examples are at hand from disparate Type 1 embryos, some of which have already been touched upon. For instance, the ability of the autonomously specified skeletogenic precursors of cleavage stage sea urchin embryos to specify gut lineage identity in recombinations with pluripotential animal cap blastomeres probably reveals a normal function. Thus the skeletogenic founder cells are in contact with the prospective gut founders in the undisturbed embryo (Davidson, 1989). The experiments of Wood (1991) cited above in which right/left handedness is shifted in early *C. elegans* embryos by changing blastomere positions can be interpreted similarly. In essence this operation will cause the left-hand AB derivative (ABpl) to contact the autonomously specified P_3 cell instead of the right-hand derivative (ABpr), and contacts with the E blastomere may be reversed as well; the prediction might be that the P_3 (or its descendants) provide the ligands for the conditional specification of these AB descendants. Similarly, Priess and Thompson (1987) infer that the interactions required to specify anterior pharyngeal muscle precursors in this embryo occur with contiguous derivatives of posterior lineages that by their test, produce the more caudal pharyngeal muscle cells autonomously. As a third example, cells of the autonomously specified D quadrant lineage of *Ilyanassa* are required for the correct conditional specification of the micromere lineages that give rise to eye, foot and shell gland (reviewed by Davidson, 1986, 455–462). Autonomous specification in Type 1 embryos may *always* involve specific ligand presentation, as well as the internal institution of a cell-type-specific pattern of gene expression.

Early transcription in Type 1 embryos

This discussion of the regulatory mechanisms underlying Type 1 early development indicates that transcription must be required *during cleavage*, for expression of ligands, receptors, and gene regulatory factors or co-factors, as well as of the characteristic early sets of downstream, cell-type-specific lineage markers. In fact, in sea urchin, ascidian, molluscan (reviewed in Davidson, 1986, 126–140) and *C. elegans* embryos (Schauer and Wood, 1990), transcriptional activity has been demonstrated at early cleavage stages. Indeed, no period of transcriptional quiescence preceding a 'mid-blastula transition' has ever been observed in a Type 1 embryo.

To summarize, Type 1 embryos generate spatial patterns of differential, *cell-type-specific* gene expression by processes of *both* conditional and autonomous specification that take place *in situ* during cleavage. These processes produce a three-dimensional array of functional cytoplasmic gene regulatory domains, that are positioned in space by the canonical cleavage planes that separate lineage founder cells of differing fate. A set of criteria that in capsulized form define Type 1 embryogenesis is shown in Table 1.

Vertebrate and insect embryos differ in fundamental respects from Type 1 embryos and from one another

Variable lineage, cell migration and late gene expression in vertebrate embryos

The suite of mechanisms by which Type 1 embryos operate is not universally found. Thus the key biological characters that provide the conditions for the process of early development just outlined are in some forms of embryo absent, or are replaced by other characters. For example, no vertebrate embryo displays an invariant cell lineage. In each individual of a vertebrate species, the cleavage pattern may differ, at least after the first few divisions. More importantly, given structures are always composed of cells of different exact lineage. Even far into cleavage, cells labeled with a lineage tracer are found to give rise to a variety of different cell types, that in quantitative and qualitative detail is unique to each embryo, although majority fates can be correlated with given regions of the blastula (reviewed by Davidson, 1986, 246–268; 1990; examples of recent results obtained with cells labeled for lineage tracing by various methods include for zebrafish, Kimmel and Warga, 1986; Warga and Kimmel, 1990; Kimmel, 1989; Kimmel *et al.* 1990; for *Xenopus*, Keller, 1976; Wetts and Fraser, 1989; Dale and Slack, 1987; for chick, Stern and Canning, 1990; Stern *et al.* 1988; Stern, 1990; for mouse, Gardner and Rossant, 1979; Lawson *et al.* 1986; Turner *et al.* 1990; Walsh and Cepko, 1990). Two of the most important sources of internal structures, tissues and organs in vertebrate embryos, the mesoderm and the neural crest, are formed from pluripotential migratory cell layers or mesenchymal cells. The neural crest provides a paradigm of a vertebrate embryological process in which the fate of individual cells is in large part determined environmentally during their migration or at their final destination (see, e.g. Anderson, 1989; Bronner-Fraser and Fraser, 1989; Le Douarin, 1990). In large measure, *cell-type-specification* throughout the vertebrate embryo occurs after the general migratory dispersion and reorganization of sheets of cells into multilayered structures that is the result of gastrulation, and that continues into neurulation and beyond. Some earlier, more general specification processes as of whole germ layers or regional anlagen can be identified, e.g.

the inductive specification of dorsal axial mesoderm in *Xenopus*, which occurs between 5th cleavage and the midblastula stage (i.e. 12th cleavage; see Gimlich and Gerhart, 1984; Gurdon *et al.* 1985; Jones and Woodland, 1987; Godsave and Slack, 1991). In the chick the initial decision of which cells are to generate the embryonic mesoderm and endoderm *vs* the ectoderm is also apparently made before gastrulation (Stern, 1990; Stern and Canning, 1990). However, *cell-type-specific molecular markers of differentiated function* can be detected in vertebrate embryos only at early gastrula stages, or thereafter. Here most available data are from *Xenopus*. The earliest example is an epidermal cytokeratin, zygotic mRNA for which appears at late blastula–early gastrula stage (stage 9–10; Jamrich *et al.* 1987). Significantly, activation of this gene is an autonomous function, in that it occurs in isolated cultured cells (Sargent *et al.* 1986). MyoD also begins to be expressed at early gastrula stage in presumptive skeletal muscle (Hopwood *et al.* 1989), followed later by muscle actins (Mohun *et al.* 1984). Most known histotypic markers first appear at the end of gastulation or in the neurula stage, e.g. several CNS neural markers (Sharpe and Gurdon, 1990) or a notochord keratin marker (La Flamme *et al.* 1988).

Vertebrate embryos differ from Type 1 embryos in utilizing specification processes that are essentially *independent of lineage and that follow migratory relocation*, but they do not differ in all respects. As in Type 1 embryos, the axis of the egg is prespecified in teleost, avian, and amphibian eggs (for references see Davidson, 1990). Some autonomously specified lineages may exist in amphibian eggs, e.g. ectoderm precursors at the animal pole of the primordial A/V axis, or at the vegetal pole, the lineages that give rise clonally to endoderm. However, unlike autonomous Type 1 lineages, these endoderm precursors remain pluripotent and plastic in fate, and they may not represent truly autonomous specifications (Davidson, 1986, 250–257; Sargent *et al.* 1986; Snape *et al.* 1987; Wylie *et al.* 1987). Cytoplasm of the dorsal posterior cells of *Xenopus* embryos may also contain determinants for dorsal specification (or at least for inductive interactions that lead to dorsal specification) since this cytoplasm, if transferred to ventral blastomeres, may cause a second dorsal axis to form (Yuge *et al.* 1990). However, most cell types in all vertebrate embryos are certainly conditionally specified, and in neither zebrafish nor mammals is there any evidence whatsoever for any autonomously specified embryonic cell type, *sensu strictu*. Nor is there any axial prespecification in the mammalian egg, in which all early blastomeres up to the 8- to 16-cell stage remain totipotent (reviewed in Davidson, 1986, 515–524).

Long-range diffusible morphogens

Vertebrate embryos rely extensively on an additional mechanism that is a fundamental aspect of their method of generating their initial body plans. They appear to operate spatially crude *regional identification functions* that initially specify major parts, and thus inform immigrant and resident cells that they are to be part of a future head, posterior region, trunk, or dorsal or ventral part of the body. At this level, *regional identification* is distinct from *cell type specification*, and must be, since the same cell types appear in all regions. Muscle, or CNS neuroblasts, are formed in head, trunk and tail, for instance, and originate from very many different variable lineages. In early Type 1 embryos, this distinction does not apply, since the initial embryo plan is directly a consequence of the relative positions occupied by the given *differentiated cell types* specified in cleavage, e.g. muscle or gut in *C. elegans*, and aboral ectoderm or skeletogenic mesenchyme in sea urchins. Classical and modern studies show that following the establishment of the initial biaxial anisotropies in the early embryo, the regional identification processes of postgastrular vertebrate development are inductive. Regional specification is mediated by *diffusible intercellular morphogens*, and at least some of the morphogens have been identified in amphibian, avian, and mammalian embryos. These are members of the FGF, TGFβ, and *Wnt* growth factor families, and retinoic acid (e.g. Kimelman and Kirschner, 1987; Durston *et al.* 1989; Mitrani *et al.* 1990; Ruberte *et al.* 1990; Smith *et al.* 1990; Green and Smith, 1990; Thomsen *et al.* 1990; van den Eijnden-Van Raaij *et al.* 1990; Cooke and Wong, 1991; Ruiz i Altaba and Jessell, 1991; McMahon and Moon, 1989). Within the inductively specified regions such as neural plate, head, and dorsal and ventral mesoderm, all of which are canonical features of neurula stage vertebrate embryos, there *then* begin to operate the postgastrular morphogenetic programs by which specific structures and tissues are progressively generated, as we discuss below.

The characters general to the vertebrate embryonic process are a distinct set, and this brief sketch suggests that at the level of mechanism vertebrate embryos 'work' in a distinct manner, with respect to Type 1 embryos. For ease of communication, in the following I refer to the vertebrate embryonic process as *Type 2*; a canonical set of characters that identify this form of embryogenesis is listed in Table 1.

Forms of insect embryogenesis

The insects display (to varying extents) a third form of basic embryonic process by which the initial spatial patterns of differential gene expression may be organized. The eggs of all orders of winged (pterygote) insects undergo intralecithal cleavage, forming a yolky syncytium. Outward migration of some of the nuclei late in cleavage gives rise to a syncytial blastoderm (reviewed by Anderson, 1973, 214–217; 1972*a*,*b*; this process also occurs in all wingless insect orders except Collembola [springtails], the eggs of which undergo several holoblastic cleavages, but then form the usual blastoderm secondarily, surrounding a yolky syncytium). Cellularization of the pterygote blastoderm occurs as membranes wall off the nuclei from one another and from the yolk mass, which is ultimately incorporated in the midgut. There is no possibility of cellular interaction while the embryo is syncytial, and

Table 1. *External embryo characters used for type classification*

Type 1
- —Invariant cleavage giving rise to canonical lineage; generally invariant blastomere fate assignments.
- —One embryonic axis prespecified in oogenesis; egg initially radially symmetrical with second axis specified after fertilization.
- —Some lineage founder cells specified autonomously at poles of embryonic axes; others specified conditionally.
- —Regulative capacity in experimental circumstances, according to amount of conditional founder cell specification in undisturbed embryo.
- —Cell type specification precedes any large-scale embryonic cell migration, and occurs *in situ* where the founder cells arise during cleavage.

Type 2
- —Variable cleavage, no canonical lineage or cell fate assignments possible; variable 'salt and pepper' specification pattern within lineage elements.
- —One (or no) egg axis prespecified in oogenesis; second (or both) specified during development.
- —Specification largely conditional; rigid autonomous specification rare or absent.
- —Capacity for regulative reorganization of large elements of body plan.
- —Massive cell movements or migrations precede or accompany specification.
- —Early regional inductions define major initial embryonic body plan.

Type 3
- —Variable syncytial cleavage; no canonical lineage; cellularization follows cleavage and blastoderm formation.
- —Both axes of egg prespecified in biaxial ovariole, and used to generate both A/P and D/V pattern elements of embryo.
- —Extensive regional specification of body plan precedes cellularization.
- —Conditional specification of cell type and of local pattern elements follows cellularization.

after cellularization the subsequent cell lineage is variable (e.g. see Beer *et al.* 1987; Technau *et al.* 1988; Technau and Campos-Ortega, 1986). The body plan becomes morphologically visible after gastrulation, with the formation of the segmented germ band along the future ventral surface of the larva, in which neuroblasts and mesoderm lie within the epidermal precursors that will give rise to the larval cuticular pattern. The fate maps of all pterygote embryos at the completed germ band stage are remarkably homologous, from primitive orders such as dragonflies to the Diptera (Anderson, 1973, 209–225, 249–253). However, the manner in which the germ band is formed differs greatly amongst the different pterygote orders (reviewed by Sander, 1976). It is largely this variable *initial process* with which we must be concerned, for it is this process that specifies the body plan of the organism; that results directly in the spatial disposition of differentially functioning cells; and that is comparable with the stages of Type 1 and Type 2 development that we considered above. In 'long germ band' insects, e.g. the Diptera, the whole metameric body plan is established more or less simultaneously by subdivision and diversification of the blastoderm. We know that in *Drosophila* the major head, tail, thoracic and abdominal regions, the future metameric pattern, and dorsoventral diversification, are already foreshadowed in differential patterns of regulatory gene expression by the time cellularization is complete (reviewed by Akam, 1987; Scott and Carroll, 1987; Ingham, 1988). 'Intermediate germ band' insects, examples of which include dragonflies and some beetles, also form the head and thoracic segments of the germ band by direct subdivision of the blastoderm, but most of the abdominal segments are elaborated *sequentially* from a posterior 'growth zone' that contains the anlagen for the posterior ectoderm and mesoderm. In further contrast, insects from several lower hemimetabolous orders (e.g. some beetles, grasshoppers, termites, mantids) initially form a 'short germ band,' in which all segments posterior to the antennal region of the head are generated from a posterior growth zone. Among the best known short germ band examples is the grasshopper (Orthoptera). Development from a growth zone is a very different process from specification of the blastoderm prior to cellularization, in that it appears to depend explicitly on intercellular interaction (Sander, 1976; Sander and Lehman, 1988; Tear *et al.* 1988). It is interesting that the eggs of long germ band insects are formed by meroistic oogenesis (Sander, 1976), i.e. in an ovariole in which most of the oocyte transcripts derive from polyploid nurse cells of germ line origin (reviewed in Davidson, 1986, 307–326), while the ovarioles of short germ band insects lack nurse cells, and at a much slower rate, synthesize their own transcripts. Recent molecular data provide a striking demonstration of the argument that despite the basic contrast in their modes of formation, *once completed*, the embryos of long and short germ band forms are functionally homologous. Thus, for example the spatial patterns of expression of the *AbdA* gene is the same in the abdominal segments of a grasshopper, *Schistocerca*, as in those of *Drosophila* (Tear *et al.* 1990); and the metameric expression of the *engrailed* gene is also the same in the embryos of these species (Patel *et al.* 1989*b*).

Conditional specification in Drosophila

In *Drosophila* many of the morphogenetic processes that follow cellularization depend on intercellular interactions, as in all embryos. For example, the differentiation of individual epidermal cells that generate cuticular pattern within each metamere is under the control of an intercellular signalling system mediated by 'segment polarity gene' products (Botas *et al.* 1988; Martinez-Arias *et al.* 1988; Hidalgo and Ingham, 1990). Among these genes, for example, *wingless* encodes a secreted protein that probably functions as a signalling ligand (Cabrera *et al.* 1987; Rijsewijk *et al.* 1987); *patched* encodes a transmembrane protein that might interact with the receptor for the *wingless* protein (Hooper and Scott, 1989; Nakano *et al.* 1989; Hidalgo and Ingham, 1990); and others encode transcription factors that likely control the presentation of relevant ligands and receptors. As another example, the product of the *decapentaplegic (dpp)* gene is a member of the TGFβ family of developmentally active ligands (Pad-

gett *et al.* 1987), and is required for cells to express dorsal embryonic fates (Irish and Gelbart, 1987; St Johnston and Gelbart, 1987), as well as for inductive functions in midgut morphogenesis (Reuter *et al.* 1990). A third example is the set of genes required for neuroblast formation within the ventral neurogenic region of the cellular blastoderm, which has been demonstrated to be a conditional specification process that also depends on intercellular signalling (reviewed in Davidson, 1986, 271–280). Among the required 'neurogenic' genes are *Notch* and *Delta*, which both encode apparent transmembrane proteins with extracellular domains displaying homology to mammalian EGF, and which may mediate intercell interactions by interacting with one another (Artavanis-Tsakonas, 1988; Campos-Ortega, 1990; Johansen *et al.* 1989; Fehon *et al.* 1990; Hartenstein and Posakony, 1990). Though genetic evidence provides a somewhat different slant on these processes than did, say, the experiments of Hörstadius and Spemann and their schools on sea urchin and amphibian embryos, molecular biology suggests that embryonic conditional specification is essentially similar throughout Metazoa, except that sometimes the ligands may be cell bound and sometimes diffusible (see Davidson, 1990). It is the processes of regional nuclear specification that occur *before cellularization* that distinguish embryogenesis in *Drosophila*, and by implication in at least other long and intermediate germ band insects (the same processes could be used in the head regions of short germ band insects as well).

Precellular processes by which spatial patterns of gene expression are established

Networks of regulatory gene interaction, which have different features, and which of course interlock (e.g. Carroll *et al.* 1987; Strecker *et al.* 1991), specify the anteroposterior metameric organization, the head and tail regions, and dorsoventral organization of the *Drosophila* egg, all to at least some extent prior to the cellularization of the blastoderm. The primordial A/P polarity of the embryo is reflected in a declining A to P gradient of the maternal *bicoid* transcription factor (Nüsslein-Vollhard *et al.* 1987); this is interpreted directly at the level of regulatory DNA-protein interactions by the zygotically expressed 'gap genes' so as to set up a series of broad overlapping domains of the transcription factors produced by the gap genes; and their local concentration clines and ratios are in turn 'read' in the syncytial blastoderm nuclei to produce transverse premetameric 'stripes' of expression of 'pair-rule' genes such as *eve*, *runt* and *hairy*. These genes then control one another, as well as downstream pair-rule genes such as *ftz* and the segment polarity signalling system (see reviews of Scott and Carroll, 1987; Ingham, 1988; Reinitz and Levine, 1990). The homeotic genes are also initially controlled by gap gene products, by pair-rule gene products, and later by segment polarity gene products (Scott and Carroll, 1987; Carroll *et al.* 1988; Harding and Levine, 1988; Ingham, 1988; Reinitz and Levine, 1990). A revealing mechanistic insight derives from analyses of the $\geqslant$8 kb *eve* regulatory domain (Goto *et al.* 1989; Stanojevic *et al.* 1989; Jiang *et al.* 1991; Small *et al.* 1991), which show that there are separate regulatory subdomains responsible for generating different stripes, and that each subdomain consists of clusters of multiple positively and negatively acting target sequences for gap gene and other transcription factors, including that encoded by *eve* itself. This is an integrating regulatory system that resolves multiple inputs into a single scalar function, transcription. Since it evidently depends on cooperative factor–DNA interactions it is exquisitely sensitive to factor concentrations in each nucleus, and thus to the shape of the gap gene product concentration profiles and ratios along the A/P axis (Small *et al.* 1991). The *hairy* gene is evidently regulated similarly (Howard and Struhl, 1990). The relevant point here is that these interactions take place in a syncytium, and the nuclei *communicate by the concentration clines of their diffusible macromolecular regulatory products*, rather than by intercellular interactions (Akam, 1987). In the head and tail are found other variations. At the anterior end, different gap genes encoding transcriptional regulators are activated by the *bicoid* factor before cellularization and may directly control segment polarity genes (*viz*, *ems*, *otd*, and *btd*; Dalton *et al.* 1989; Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990). In the head and the tail, furthermore, a uniformly distributed maternal receptor tyrosine kinase, the product of the *torso* gene, is regionally activated, apparently by a ligand presented on the terminal follicle cells. The signal transduction system that it controls regulates nuclear expression, again before cellularization, of various downstream genes required for terminal differentiation (including the gap genes *tailless* and *otd*; Casanova and Struhl, 1990; Sprenger *et al.* 1989; Finkelstein and Perrimon, 1990; Pignoni *et al.* 1990; Strecker and Lipshitz, 1990; Strecker *et al.* 1991). Still a different precellular regulatory system controls dorsoventral pattern formation. Here the key transcription regulator is *dorsal* (Ip *et al.* 1991). The presentation of *dorsal* is mediated along the D/V axis by a localized cytoplasmic machinery, generated by the products of a number of maternal genes required for dorsoventral specification, that acts to modify the cryptic *dorsal* regulator so that it is transported into the cell nuclei only on the ventral side of the blastoderm (Levine, 1988; Steward, 1989; Roth *et al.* 1990; Rushlow *et al.* 1989). The downstream targets of the *dorsal* regulator are again genes encoding other transcription factors, as well as the *dpp* gene (Ip *et al.* 1990).

Thus there is a variety of distinct mechanisms utilized to achieve precellular spatial pattern of expression. By means of gene interactions, following the prelocalized biaxial maternal spatial cues, these mechanisms essentially accomplish what Type 1 and Type 2 processes accomplish by entirely different mechanisms, which are not available to the syncytial stage *Drosophila* embryo. For convenience the processes exemplified in the early *Drosophila* embryo are referred to as *Type 3* embryogenesis in the following, and can be summarized in

outline as in Table 1. Their end result is of course the same as of Types 1 and 2 processes, *viz,* generation of an assembly of spatially organized cells, according to the body plan, that can now mount the intercellular functions required for cell-type-specification and morphogenesis.

Phylogenetic distribution of embryo types

A speculative extension of typological classification, and ubiquity of Type 1 process

Thus far we have been concerned with 'model systems,' i.e. with relatively well studied embryos, which we assume are archetypical for their respective taxa. In considering other metazoan groups, the available information is rarely adequate. Relatively little molecular biology, developmental genetics, or experimental embryological manipulations such as might directly reveal the nature of specification processes are to be encountered outside the 'model systems.' In as much as each type of embryonic process can be defined by an easily observed set of external characters, however, it is worth trying to assess the available descriptive and experimental data so as to arrive at a more inclusive idea of the phylogenetic distribution of these Types. Table 1 summarizes in brief form the sets of external criteria that I have used in this speculative exercise, and Fig. 1 shows its result. The embryological evidence, and references used for these characterizations are very briefly indicated in Notes to Fig. 1, except for the groups whose relatively well-known representatives were considered above, and in Davidson (1990).

Fig. 1 is organized as a phylogenetic chart of Metazoa, omitting several minor phyla of which relatively little is known. Superimposed on the chart is a color code indicating embryo type for each phylum; blue for Type 1, red for Type 2, and yellow for Type 3. Phylogenetic aspects of the chart are described in the legend. There is no universally or perhaps even generally accepted phylogenetic organization for such a diagram, because of the uncertainties surrounding the adult and embryonic morphological homology arguments, and the evolutionary and paleontological interpretations, on which it must be based. However, the particular arrangement shown in Fig. 1 is not very important for our present purposes, given its rather simple and direct import. This is that Type 1 embryogenesis is the basic, typical mode of early development in almost every invertebrate phylum. In many of these phyla certain orders or families or even particular genera display significant deviations from the canonical Type 1 process (see below), but only embryo types that appear to possess *Order*, *Class* or *Phylum* levels of generality are indicated by the color code in Fig. 1. At this level Type 2 embryonic process is characteristic only of vertebrates, and possibly of the cephalopod molluscs and the hydroid cnidarians. Type 3 embryonic processes occur in long germ band orders of winged insects, and most probably in intermediate germ band orders. This includes wingless as well as winged insects. It is important to note that uniramian embryos of all classes, e.g. myriapods as well as insects, display some version of intralecithal or syncytial cleavage (see legend to Fig. 1 for references and further details).

Fig. 1 implies a fascinating evolutionary argument, *viz* that the *original form of embryogenesis*, which must have appeared before the Cambrian, was and is Type 1 embryogenesis. Other forms of embryonic process might therefore be regarded as derivations of Type 1 embryogenesis. Note in this connection that the oldest known fossil true vertebrate, a jawless fish, appears in the Upper Cambrian; an acraniate is known from the Middle Cambrian and the oldest uniramian ancestors also appear to have arisen in the course of the Cambrian, though the earliest myriapods and wingless insects appear only in the Devonian. The earliest true coelomate protostome forms are already present at the beginning of the Cambrian, however, and animals similar to the acoel and pseudocoel protostomes of today are thought to have existed in the pre-Cambrian (see, e.g. Manton, 1977, 26–27; Richards and Davies, 1977, 421–427; Valentine, 1989; Jeffries, 1986, 333–335; Kuhn-Schnyder and Rieber, 1986). It thus becomes instructive to consider what regulatory changes might we imagine by which Type 2 and Type 3 embryonic process might have derived from their respective deuterostome and protostome ancestors.

Direct developing Type 1 variants

A clue perhaps emerges from minor phyletic variants in embryonic process that are not indicated in Fig. 1. The Type 1 groups shown in this Figure characteristically develop indirectly, in that the business of the embryo is to produce a feeding larva, which in turn provides a life support system for more complex morphogenetic processes required to generate the juvenile form of the adult. However, in many of these phyla there occur variants that develop the juvenile form directly. These are undoubtedly derivative variants of the canonical, indirectly developing forms in each phylum (e.g. for annelids, see Anderson, 1973, 88–90; for echinoids Raff, 1987; for ascidians Jeffery and Swalla, 1990). For instance, in the sea urchins, about 20% of the approximately 200 studied species show some variation from the canonical form of indirect development, and direct development has arisen *independently* in six of the ten echinoid orders. Sometimes, as in both echinoids and ascidians, the *same genus* will include species that develop directly, and species that develop indirectly. This shows that the types of evolutionary change leading to direct development can occur relatively easily, in evolutionary, and in regulatory terms. The kinds of regulatory change that have occurred include deletion of regulatory programs; heterochronic changes, in which developmental processes that are used in larval morphogenesis in indirectly developing forms are instead used in embryonic development; and other qualitative inventions. For instance, in directly developing ascidians, there is an

alteration in the localization of maternal determinants required to specify gene expression in the larval tail muscle, and the expression of embryonic muscle genes is deleted (Jeffery and Swalla, 1990; Swalla *et al.* 1991). Changes in the timing and order of internal organ system morphogenesis, in stage of founder cell specification, and in timing of expression of lineage-specific marker genes (Parks *et al.* 1988; Raff *et al.* 1990; Wray and Raff, 1990) are among the differences that distinguish direct development in sea urchins from typical indirect development. Indeed heterochronic (and other) changes in lineage specific gene expression can be observed even in comparing various indirectly developing sea urchin species (Wray and McClay, 1989). For the most part, species belonging to typically Type 1 phyla, which display direct development, nonetheless retain a Type 1 form of embryonic process, by the external criteria of Table1. Examples include the direct developing ascidian species cited above; and the direct developing oligochaete and leech classes of annelids. These embryos fulfill Type 1 criteria as well as do the archetypical marine polychaete annelid embryos, despite their often enormous, yolky eggs, and altered forms of spiral cleavage (Anderson, 1973, 51–90; references and summary in Notes to Fig. 1, where examples from other phyla are also indicated). However, what is of particular interest here is that in some of the independent explorations of direct development that have occurred frequently in evolution, variations appear that seem to foreshadow Type 2 or Type 3 embryonic process. For instance, early development in the order Hydroida, of the cnidarian class Hydrozoa, has many features that are amazingly reminiscent of Type 2 development (reviewed by Freeman, 1983), though other hydrozoan orders display Type 1 development (as do ctenophore embryos; see Fig. 1, and Notes to Fig. 1). Thus, in Hydroida cleavage is variable and there is no canonical lineage; regional organization is evidently mediated in part by diffusible morphogens, as well as shorter range interactions; all early blastomeres are totipotent; and the whole early body plan can be regenerated from pieces that normally would have formed only ectoderm or endoderm (reviewed by Freeman, 1983, 1990). In a direct developing sea urchin, as another example, the lineages of vegetal cells have become variable, while the animal cell lineages remain invariant and similar to those of typical indirect development (Wray and Raff, 1989). Similarly, in the large, yolky eggs of some decapod crustacea (i.e. shrimps, lobsters and crabs), spiral cleavage is lost or obscured; some syncytial cleavage is observed in the presumptive midgut region of the egg; and a blastoderm-like layer of cells forms around the outside of the yolk mass (Anderson, 1973, 278–282); these features are reminiscent of Type 3 development. Adaptive advantages of various forms of direct development have evidently led to the repeated derivation of these kinds of changes. Thus the major incidences of Type 2 and Type 3 embryogenesis shown in Fig. 1 may represent only the phyletically more important fixations, and extensions, of processes that have started to occur many times over in diverse evolutionary branches.

Specific downstream functions of regionally expressed homeodomain regulators in later vertebrate development

Irrespective of the deep distinctions amongst various types of biological embryonic process, at the level of the molecular biology of gene regulation they all seem at first glance the same. All metazoan embryos probably use zinc finger regulators, various homotypic and heterotypic pairs of helix-loop-helix regulators, and regionally expressed homeodomain regulators during development. These could be regarded merely as common building blocks of which diverse regulatory structures are constructed, but homologies between distant developmental processes extend to higher levels of organization as well. Our field has recently been absorbed for example, by the evidence suggesting that homologous, evolutionarily conserved clusters of homeobox genes might be utilized for anteroposterior developmental processes throughout the bilateral metazoans. The regional spatial expression of homeodomain regulators in all forms of developing animals, and the homeotic morphological effects of gain-of-function regulatory mutations in some of the genes encoding these regulators in *Drosophila*, make the functional role of these genes a particularly interesting issue. We now have a relatively good understanding in *Drosophila* of how their mutual cross-regulatory interactions are utilized in setting up their own spatial domains of expression within the organism. The general nature of the downstream targets of regionally expressed homeodomain transcriptional regulators has so far proved an elusive experimental objective. It is clear, however, that the general problem of how spatial patterns of gene expression are coordinated in embryogenesis intersects with the problem of homeodomain regulatory function. In the following I explore a general interpretation of the downstream *biological functions* of these elements of the developmental regulatory system, and then briefly reexamine the differences – and similarities – in the regulation of spatial patterns of gene expression in the different forms of embryogenesis.

What do regionally expressed vertebrate homeodomain regulators actually control?

A great deal of descriptive evidence has also accumulated regarding the patterns of expression of homeobox genes in vertebrate development. Some of this evidence, considered in conjunction with the biological character of vertebrate developmental processes, suggests a specific functional role for homeodomain regulators, at least in vertebrate ontogeny. We are here concerned only with those homeodomain regulators that are expressed *regionally* during vertebrate development, in the specific sense that they appear in a given spatial domain of the body plan, but in a variety of different cell types within that domain. This *excludes*

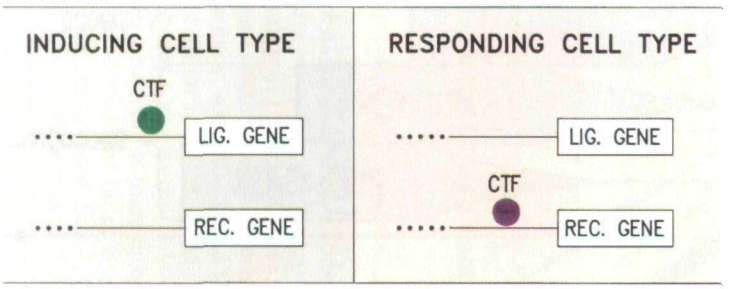
Fig. 1. Phylogenetic chart of Metazoa, with embryo type indicated in color (blue, Type 1; red, Type 2; yellow, Type 3).

Phyletic Aspects: It is impossible to cite a phylogenetic tree, or a cladogram, that is not in some measure and in some regions controversial (see, e.g., Willmer, 1990). The chart is divided horizontally into three main regions. As indicated on the right margin all groups above the bottom dashed line have nerves and mouth; above the second dashed line all groups have bilateral symmetry and mesoderm; above the third dashed line all groups have mesodermal coeloms or coelomic compartments. These are body cavities lined with mesodermal sheaths (e.g., peritoneum) so that internal organs are not situated directly in the body cavities, but rather behind mesodermal boundaries (that are often of major developmental importance). The overall geometrical design of the chart, the angles at which the various lines intersect, and the lengths of the vertical and horizontal stems that connect these lines to the current phyla shown, are entirely arbitrary design features, and have no biological significance. The diagram shown here is particularly based on the following sources: the division of arthropods into crustacean and uniramian phyla reflects the conclusions of Anderson (1973) with respect to arthropod embryological forms; of Manton (1977, 236–291) regarding arthropod structure; and the paleontological assessment of Valentine (1989). The chelicerates (e.g., horseshoe crabs, spiders, ticks) are omitted, since there is little relevant experimental embryological information. The molluscs are not considered fundamentally metameric (e.g., see Barnes, 1980, 462), and like the annelids and the arthropod phyla, can be regarded as descendant from an ancestral assemblage of spirally cleaving, protostomial ancestors. However, neither group is regarded as derived *from* annelid ancestors. The tentaculates (phoronids, bryozoa, brachiopods) possess some deuterostome characteristics, e.g., their trimerous coeloms, the mesoderm of which arises by a similar process of delamination and outpocketing from the archenteron as in echinoderms and other deuterostomes. However, they also display protostome characteristics, e.g., formation of mouth from the site of blastoporal invagination. Thus the shaded areas indicating the regions occupied by the Deuterostomia and Protostomia, respectively, *overlap* in the tentaculate area of the chart. The relation of nematodes (and related minor phyla) to the contiguous groups, flatworms and nemerteans, is uncertain, since while flatworms and nemerteans both display obvious spiral cleavage, nematodes have a different characteristic cleavage pattern, but on the other hand they share with nemerteans a pseudocoel body organization (mesodermal body wall but no mesodermal layer surrounding the gut or other visera, so that the body cavity is not enclosed in mesoderm). Flatworms (acoel grade) have mesoderm but neither coelomic nor pseudocoelic cavities. The ctenophores (e.g., comb jellies) and cnidaria (jellyfish, anemones) are radially symmetric animals, and have no true mesoderm. For the deuterostome branch I have followed Jeffries (1986) in placing hemichordates as the most primitive living true deuterostome representatives, and as such the sister group of all remaining deuterostomes, though this view remains controversial. Echinoderms, tunicates (e.g., ascidians), acraniates (e.g., *Amphioxus*), and vertebrates can all be considered 'dexiothetes'; the term refers to the development of organ systems from coelomic domains that are homologous to the left coeloms of hemichordates, as if in ancestral deuterostomes of hemichordate grade the right side had become ventral and the right coeloms and other structures had disappeared (Jeffries, 1986, 52; Jeffries, 1990; Paul, 1990; Willmer, 1990, 319–326); note that it would be of little import for the main purposes of this Figure were the positions of hemichordates and echinoderms reversed. Acraniates have been placed as the sister group of vertebrates rather than tunicates, on the basis of the distribution of a large number of shared characters amongst these groups, as enumerated by Maisey (1986) and Schaeffer (1987), for example. Maisey (1986) and Jeffries (1986, Ch. 5–6) and the earlier authorities whose works they review, have assembled a large number of characteristics shared by all true vertebrates that cannot be enumerated here, e.g., appearance of genuine neural crest, olfactory and optic capsules of brain, somitic structures, etc. Examples of prominent characteristics important for the organization of the diagram that appear in *particular* phyletic branches, are indicated across the stems leading to the modern groups; of course these are merely indicative, in each case, of a large number of definitive characters. Proceeding upwards along the protostome and deuterostome branches the characters indicated by the black thin lines parallel to the heavy green main branches are shown cumulatively; thus for example, on the protostome line, from the ancestors of the nemerteans up, all groups descended from ancestors with hemocoels (fluid filled body cavities not lined with coelomic mesoderm), and from the ancestors of annelids up, all groups descended from metameric ancestors (i.e., animals composed of repetitive homologous body segments). These are selected from among the nested sets of shared characters that support the order shown on the phyletic stems.

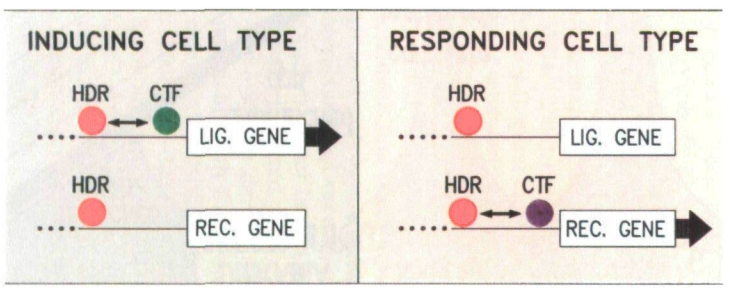
Embryological Aspects: The classifications indicated in color are based on the criteria summarized in Table 1 and discussed in text. Adequate experimental data are in some cases not available, particularly with respect to the key parameter, the initial mode of embryonic cell fate specification. In these cases the phyla are indicated by striped rather than solid color patterns. For relatively well studied nematode, dipteran, gastropod and other molluscan, echinoderm, ascidian, amphibian, teleost, and mammalian embryos the assignments of embryo type are based on data summarized in Davidson (1990), and review of relevant information in Davidson (1986, Ch. 4 and 6). Evidence supporting the Type 1 assignments shown for cnidarian, ctenophore, nemertean, crustacean, annelid, tentaculate, hemichordate, and acraniate embryos is very briefly cited in *Note to Fig. 1.* The classification obviously rests on observations on a few type species of each group that have served as objects of investigation, and there may be many deviant embryological modes, particularly among direct developing species, or species with very yolky eggs (see discussion in text). These, however, are generally regarded as derivative forms. For the Uniramia, Anderson (1973) provides a key comparative treatment of hexapod, myriapod (i.e., millipedes, centipedes, etc.), and onchyphoran embryos. Very little detailed experimental information exists that in fact supports the generalization of Type 3 mechanisms outside the long and intermediate germ band orders of insects (see text). Myriapod embryos could be described as intermediate germ band forms in

2A

① EARLY DEVELOPMENTAL ESTABLISHMENT OF CELL TYPES

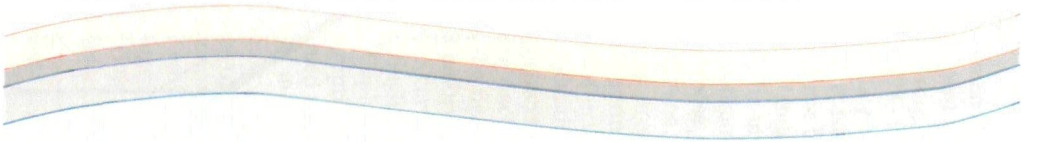


② REGIONAL SPECIFICATION AND INDUCTIVE CELL INTERACTION

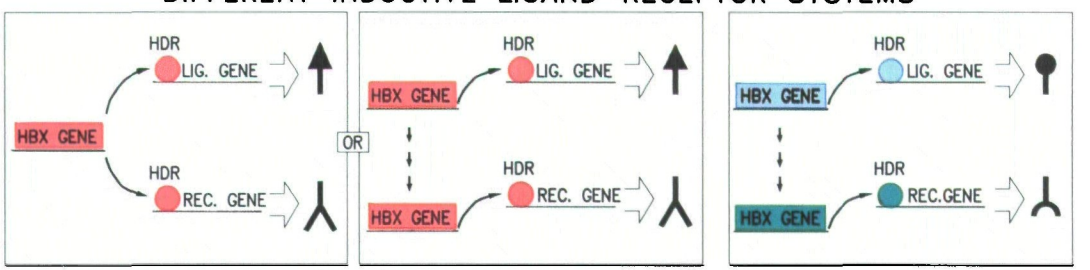


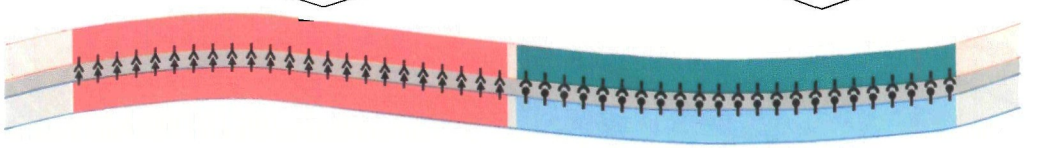
B

① EARLY DEVELOPMENTAL ESTABLISHMENT OF CELL TYPES



② REGIONAL EXPRESSION OF HBX GENES AND ACTIVATION OF DIFFERENT INDUCTIVE LIGAND–RECEPTOR SYSTEMS





SIGNAL TRANSDUCTION MEDIATED GENE EXPRESSIONS & CELL INTERACTIONS DETERMINING 3D PATTERN

MORPHOLOGICAL STRUCTURE

MORPHOLOGICAL STRUCTURE

recombinations no respecification of blastomere fates along the animal–vegetal axis can be detected (Hörstadius, 1937). The egg is polarized in this axis when shed. However, isolated 1/2 eggs, including, e.g., both 1/2 blastomeres of one egg, generate normal bilaterally symmetrical larvae, as do at least some 1/4 eggs (Wilson, 1903; Hörstadius, 1937). This clearly requires regulation and hence in normal embryos, conditional specification, at least along the second (A/P) axis of the egg.

Crustacea: These are basically spirally cleaving eggs, as can be seen, for example, in barnacles (reviewed by Anderson, 1973, 268–280 and Green, 1971, 320–333). Yolky crustacean eggs display adaptive modifications, including radial cleavage, sometimes involving secondary features such as generation of a 'blastoderm' covering the yolk. In contrast to insects, canonical invariant lineages have been described for many crustacean species (Kumé and Dan, 1968, 341–352). The fate map of the spirally cleaving forms is decisively different from that of annelids, but is homologous in all crustacean forms (Anderson, 1973, Chapter 8). There is little detailed modern evidence on the nature of blastomere specification processes. However, germ cells are probably autonomously specified by maternal determinants in copepods and other groups (reviewed by Wilson, 1925, 314). The egg is evidently polarized in the initial An/Vg axis; and mesoderm derives from specific founder cells (3A, 3B, 3C), as does the gut, though the lineage differs amongst orders (Anderson, 1973, *op cit*). These founder cells are likely to be autonomously specified, as in other spirally cleaving forms (though the fate of individual lineage elements is strikingly different from that in the annelid spiral cleavage map). On the other hand, regulative capability has been reported (Green, 1971, *op cit*). Furthermore in crayfish posterior segments develop progressively from a 'growth zone,' as in intermediate or short germ band insects, and these segments express *engrailed* at their posterior boundaries (Patel *et al.* 1989*a*), suggesting a homologous process that depends on intercell interaction for posterior segment formation. The assumption that crustaceans have Type 1 eggs is tentative, though it seems unlikely to be incorrect.

Annelids: Polychaete annelid embryos exemplify canonical invariant spiral cleavage, and have a canonical fate map (for recent review see Anderson, 1973). Oligochaetes that have yolky eggs display certain modifications in cleavage pattern but homologous fate maps. Many classical studies demonstrate autonomous specification of both oligochaete and polychaete annelid lineages. Among these are mesoderm and the ectodermal neuroblast germ band lineages, apical tuft, specific bristle and protocyte lineages. Such results have been obtained in experiments in which individual blastomeres or specific groups of blastomeres are killed, raised in isolation, or transplanted to ectopic positions; see experiments of Penners on *Tubifex*, (reviewed by Morgan, 1927, 370–380), of Novikoff (1938) and of Render (1983) on *Sabellaria*; of Henry (1986, 1989) and Henry and Martindale (1987) on *Chaetopterus*. Furthermore, partial double embryos that do not regulate to form a single larva are produced *in situ* by equalization of cleavage in *Tubifex* (reviewed by Morgan, 1927, 412–417), and partial twin embryos are generated by equalization of cleavage in *Chaetopterus* (Henry and Martindale, 1987). All of these results imply autonomous specification by maternal determinants localized in given lineage founder cells (Davidson, 1990). However, in the twin studies on *Chaetopterus* of Henry and Martindale (1987), it is clear that, to account for the morphological results, the D blastomere must exercise an inductive effect on C blastomere derivatives as well as giving rise to autonomously specified structures. In direct developing annelid forms exemplified by the leech *Holabdella* (Class Hirudinea) autonomous specification of the 'mesoblast' stem cell (in fact this also gives rise to a few neuroblasts; Weisblat *et al.* 1984), and of the neuroectoteloblast, is demonstrated by centrifugation experiments. These show that supernumerary founder cells develop when a visible maternal cytoplasm (teloplasm) is distributed to the C as well as D blastomere (Astrow *et al.* 1987). However, several studies also show that in this and other leeches the founder cells for several neuroblast lineages are conditionally specified, so that their identity depends on that of their neighbors (Weisblat and Blair, 1984; Keleher and Stent, 1990). Annelid embryos thus have all the characteristics of Type 1 embryos; a monopolar egg organization *ab initio*; invariant cleavage; and utilization of both autonomous and conditional founder cell specification processes.

Phoronida: Little is known of the cellular embryology of tentaculates outside Phoronida. The egg is apparently preorganized along the A/V axis. The animal pole is clearly marked by polar bodies and by egg shape, which is flattened at A and V poles. The first two planes of cleavage are orthogonal in this axis, the third transverse, just as in hemichordates and echinoderms (Kumé and Dan, 1968, 242–245; Zimmer, 1964; Emig, 1977). In cleavage two radially segmented tiers of eight cells each are formed, and then four tiers (as also in other tentaculate phyla, *viz* ectoprocts and brachiopods). There is no good evidence for localization of determinants along the A/V axis, except that like sea urchin embryos, the animal blastomere quartet isolated after the transverse 3rd cleavage fails to regulate, forming only a ciliated ball of cells that express none of the neuronal markers normally produced by the progeny of these cells (Freeman, 1991). However, vegetal half 3rd cleavage embryos regulate to produce whole new embryos (Freeman, 1991), while 1st and 2nd cleavage blastomeres form almost complete embryos which, however, lack normal tentacles (Emig, 1977). These characteristics are typical of Type 1 embryos, in which there is normally some autonomous specification along the A/V axis, though it is clear that neurectodermal differentiation in animal cap cells requires influences from vegetal cells. Furthermore the second axis is specified after fertilization, as in Type 1 eggs. On the other hand, anteroposterior blastomere fate specification occurs only late in development, in gastrulation, and the lineage and cleavage patterns are somewhat variable (Freeman, 1991). These are significant deviations from Type 1 characteristics. Hence the Figure displays a striped color pattern for this phylum indicating that the assignment of Type 1 embryonic process to this group is not unequivocal.

Hemichordates: Significant information exists only for some species of Class Enteropneusta (acorn worms). Eggs of *Saccoglossus* display radial cleavage of a form remarkably similar to that of echinoids; thus 4th cleavage is radial in the animal half of the embryo and horizontal in the vegetal half, etc. The egg is polarized *ab initio* in the A/V axis, which corresponds to the future A/P axis of the embryo (Colwin and Colwin, 1953). Cleavage is canonical and was followed to the 128-cell stage, but Colwin and

Colwin (1953) observed some variation in the positioning of the 4th cleavage planes, without any disturbance of subsequent development. The initial fate map along the A/P axis is similar to that of echinoderms. Thus the lowest tier of blastomeres at the 16-cell stage gives rise to gut and coeloms (like veg_2 in sea urchins) and the animal half plus the veg_1 equivalent produces ectoderm (Colwin and Colwin, 1953). Both members of pairs of isolated 1/2, and some isolated 1/4 blastomeres, form complete embryos, again as in sea urchins. Though this indicates conditional specification (at least along the A/V axis), there is no direct evidence of any autonomous specification in these embryos. However, the detailed similarities (very briefly noted here) to early sea urchin embryos suggest that early developmental processes are generally similar as well; hence their (somewhat tentative) Type 1 classification.

Acraniates (or Cephalochordates): As described by classical workers (reviewed by Reverberi, 1971), the canonical cleavage gives rise to a four-tiered, 32-cell embryo, with a more or less invariant fate map (Tung *et al.* 1962; Reverberi, 1971). Extensive blastomere tier recombinations, and other experiments in which one or another tier is deleted, demonstrate that most of the lineage fates are plastic, i.e., are in normal embryos conditionally specified (reviewed by Reverberi, 1971). The neural tube depends for its formation on contact with vegetal anterior blastomeres, and can be formed from cells that normally give rise only to ectoderm in ectopic recombinations. Similarly, ectopic founder cells for notochord are generated in chimeric recombinations. However, at least some autonomous specification capacity is elicited in cultures of isolated vegetal tiers which in some cases give rise to gut, a normal product of this tier, and embryos lacking the veg_2 tier give rise to larvae lacking the gut. In addition, pairs of isolated 1/2, 1/4 or 2/4 blastomeres form complete larvae, though animal half 4/8-cell embryos produce only ciliated balls (Wilson, 1893; Conklin, 1932; Tung *et al.* 1958). The embryo thus in many respects resembles that of the sea urchin (Hörstadius, 1939; Davidson, 1989), relying largely on conditional founder cell specification, but with an initial A/V organization reflected in some autonomous vegetal (gut) specification (in the sea urchin the vegetal-most lineage is skeletogenic, and it is this, rather than gut, which is specified autonomously).

Fig. 2. Abstract depiction of simplest versions of proposed function of regionally expressed homeodomain regulators in vertebrate development. (A) *Intranuclear regulatory requirements* if the homeodomain regulator directly activates (or represses) *both* the ligand and receptor genes of locally interacting cells (the same functional relationship would obtain if the homeodomain regulator directly activates a gene encoding a transcription factor, that in turn activates both ligand and receptor genes in the respective cell types). In 1, cells of two different layers that are destined to interact in order to produce a morphogenetic event are indicated in different colors. These cell types have been established by a prior process of specification; e.g., they could be mesoderm and ectoderm or mesenchyme and epithelium, neural crest derivatives and cells with which they will react, etc. Cell type factors (CTF) are each specific to one cell type; the ligand (lig) gene has a site for one of these factors and the receptor (rec) gene for the other. At 2, in the following stage of development the homeodomain regulator (HDR) is shown bound to a second regulatory target site in the gene coding for the inductive ligand and *also* in the gene coding for the receptor. That is, *both* genes have sites that can be productively occupied by the homeodomain regulators. Solid arrow indicates transcription of these genes, which occurs when both the CTF and HDR sites are bound, presumably by a mechanism involving factor interactions (double headed arrow). (B) *Morphological scale*. In 1, a relatively large region of the two layers (pink and tan) is indicated, separated by an extracellular matrix (gray). Later in development this region will give rise to two different morphological structures, but until regional expression of the homeodomain regulators occurs, these tissue layers are developmentally equivalent in the horizontal direction. In 2, three different regulatory circuits are shown in the boxes, representing different ways in which coordination of regional expression of ligands and their receptors might be organized by homeobox (HBX) gene expression. On the left, as in part (A) of the Figure, a homeobox (HBX) gene regulates its ligand in one of the early cell types and its receptor in another; this gene, and its HDR product are shown in red. The gene might have been turned on in both layers by certain levels of a diffusible morphogen, for example. An alternative is shown in the middle panel, in which the homeobox gene controlling the expression of the ligand in the inducing cell also makes use of a preexistent signalling system in the responding cell type to induce its own expression. This then results in the production of the specific *downstream* interaction system symbolized by the ligand and receptor, that will mediate morphogenetic development (were the preexistent signalling system adequate to mediate morphogenetic development itself, homeobox gene expression in the responding cell would not be required, nor presumably, observed, unless the same molecule acts as ligand and receptor). In the right box two different homeobox genes are active (genes and products in blue and turquoise). The top gene controls the ligand and the bottom the receptor, as shown. The genes are coordinately activated in the different cell types either by an internal regulatory network that depends in part on cell-type-specific factors as in A; or by an inductive activation as in the middle box. Different homeodomain regulators might be required because their signal and receptor functions are utilized separately elsewhere in the organism. Of course innumerable more complex (and no doubt more realistic) variants can be imagined than those shown in this cartoon: e.g., there may usually be needed not one but several homeodomain regulators in certain particular concentrations; or the receptors could be constitutive in some cell types and not be regulated; or the cell-type-specific transcription factors shown in A could be several factors, some controlling temporal expression, some responding to signals from other cells; etc. Below, the presentation of two different sets of *morphogenetic ligands and receptors* is indicated, resulting in two different morphological processes. The different homeodomain proteins expressed in the red, and in the blue and turquoise, region mediate different modular ligand–receptor interactions, producing spatially separate, diverse structures.

the cell-type-specific homeodomain regulators of vertebrates, of which the downstream targets are indeed genes encoding cell-type-specific products, e.g. Pit1 in rat pituitary cells (Ingraham *et al.* 1988); HNF-1 in the rat liver (Baumhueter *et al.* 1990); Oct-2 in human B cells (Clerc *et al.* 1988); and perhaps the product of the mouse *Cdx-1* gene, which is expressed only in differentiating intestinal epithelium cells late in embryogenesis and in adults (Duprey *et al.* 1988). Note that the same homeobox genes can be expressed regionally at one stage in development, while at a later stage they may have a cell-type-specific role elsewhere in the organism (e.g. Vogels *et al.* 1990); we are here concerned only with the circuitry and function of such genes when they are being expressed regionally. In amphibian embryos regionally expressed homeodomain regulators first appear during mid–late gastrulation, *after the initial broad specification of the major regions of the embryonic body plan has already occurred, or as it is in process.* Many such genes then continue to be expressed in a changing spatial pattern throughout postembryonic development, particularly during morphogenetic and organogenic processes.

A simple interpretation of the developmental function of this class of regionally expressed regulatory gene, for which some evidence is discussed below, is as follows:

(i) Downstream functional role

The biological function of regionally expressed homeodomain regulators might be specifically to promote spatially organized expression of genes encoding morphogenetic ligands, receptors, particular signal transduction machinery, and extracellular components that mediate ligand–receptor interactions. The premise is that organogenesis and local morphogenesis of structure proceeds by means of short-range ligand–receptor interactions amongst different cell types, which specify the location and function of the constituent cells with respect to one another. The specific downstream targets of homeodomain regulators would thus be regulatory binding sites in these 'cell communication' genes, or perhaps in genes encoding transcription factors that specifically control 'cell communication' functions. The function of *regional* homeodomain regulator expression would therefore be to control the *coordinate* expression of the ligands and the receptors required for given morphogenetic developmental modules. This could be accomplished by control of both the ligand and the receptor system by the same homeodomain regulator (in different cells), or by different homeodomain regulators, as illustrated in a simplified way in Fig. 2 (see legend).

(ii) Upstream regulation of regionally expressed homeobox genes

Spatial expression of this class of homeobox genes in vertebrates will depend on the prior general axial specification processes, and will be regulated in part by diffusible morphogens, since these may be utilized in vertebrate embryos to specify domains in which particular morphogeneses will occur, i.e. to specify what are sometimes called 'embryonic fields' (this is discussed in the following section). In part they will be regulated by homeobox gene interactions, including interactions at the intranuclear network level, and at intercellular boundaries, by short-range inductive interactions (cf. Fig. 2).

(iii) Conditional specification

Morphogenesis proceeds by conditional specification, in the particular sense considered in the initial section of this paper, which deals with Type 1 embryos. That is, the number of inductively *competent* cells always exceed those actually utilized in a given response, and certain cells other than those actually responding to any given signal could always have responded, had they been in the right place (or were they put in the right place). The interpretation of conditional specification (or regulative capacity) given above implies that cell-type-specific genes that function as a result of morphogenetic induction, e.g. in organogenesis, are likely to be activated by selective modification of preexisting transcription factors, mediated by inductive signal transduction. *The perhaps surprising interpretation emerges that genes encoding cell-type-specific products of terminal differentiation are unlikely to be the direct downstream targets of homeodomain regulators.*

(iv) Evolutionary conservation of ligand, receptor and homeobox gene control circuits

Homeodomain regulators and the downstream ligand and receptor genes that they control are likely to constitute conserved genetic regulatory modules (rather than just the homeobox genes *per se*), since change in the DNA-binding elements of these regulators would require corresponding changes in the regulatory domains of the target genes, and *vice versa.* Thus given homeobox genes and their downstream targets can be thought of as 'regulatory kernels,' that are likely to be found in many organisms, and are likely to be used over and over within the same organism, and amongst organisms, to mediate different inductive morphogeneses. The number of different downstream target genes of regionally expressed homeodomain regulators thus may be quite limited.

Expression of homeobox genes both in inducing and responding tissues in vertebrate development

In a local morphogenetic process proceeding by way of short-range inductive intercellular interactions, the inducing and the induced tissues have in common only the rather teleological property of 'similar positional identity', and the mechanistic property of coordinate expression, respectively, of the ligands and the receptors that mediate their interaction. They do not in general express other downstream structural genes in common. Thus the experimental observation that homeodomain regulators are regionally expressed in *both* inducing and induced cells is significant. Many examples that may illustrate the principles supposed in Fig. 2B are to be found in the recent literature,

obtained by *in situ* hybridization or immunocytology. These show for various stages of mouse, *Xenopus*, or chicken development the tissue, and sometimes the cellular locations of homeobox gene transcripts, or their homeodomain protein products. A very brief account of a few pertinent examples follows. The *first of these examples* concerns regional, late embryonic morphogenesis of the CNS. Formation of the anteroposterior elements of the brain and spinal cord is probably programmed by interactions with the immediately contiguous mesodermal tissues (see Holtfreter and Hamburger, 1955; Keynes and Stern, 1988). Expression in *both* mesoderm and CNS neuroectoderm early in CNS morphogenesis is observed for many homeodomain genes in the mouse (e.g. see Gaunt, 1987, 1988; Kessel and Gruss, 1990; Murphy and Hill, 1991; see Fig. 2B, left-most box). The early expression patterns of the *Hox-2.9* gene (*labial* related) are particularly interesting. In the late gastrula of the mouse embryo, transcripts of the *Hox-2.9* homeobox gene first appear in migrating mesodermal cells that will become associated with the CNS in the posterior half of the embryo (Frohman *et al.* 1990). The anterior boundary of the position that these cells will occupy is the posterior hindbrain. By the early neurula stage that portion of the neuroectoderm overlying the expressing mesodermal cells also transcribes *Hox-2.9*, sharing the same anterior boundary of *Hox-2.9* transcription with the expressing mesoderm. Later the pattern sharpens and intense *Hox-2.9* expression is limited to rhombomere 4 as that structure differentiates, but the transcripts continue to be expressed in *both* mesoderm and neuroectoderm (Murphy *et al.* 1989). The initial registration of expression may be the direct result of induction of *Hox-2.9* expression in neuroectoderm by *Hox-2.9* expressing mesoderm (Frohman *et al.* 1990; cf. Fig. 2B, middle box). In chick the paralogous homeobox gene, whose expression is also ultimately confined to rhombomere4, is called *Ghox-lab*, and the mRNA, and the nuclear homeodomain protein are again found in the neuroectoderm and underlying mesoderm at the gastrula stage, even before neural plate formation (Sundin and Eichele, 1990; Sundin *et al.* 1990). A *second example* also emphasized by Frohman *et al.* (1990) concerns the expression of *Hox-2.9* not only in the branchial arch mesoderm, which derives from neural crest, but also in the contiguous endoderm of the pharyngeal pouch, and the overlying ectoderm, amongst which the anterior boundaries of expression are again all in register. The mesoderm is thought to provide short-range inductive signals that direct foregut morphogenesis (Copenhaver, 1955; Okata, 1957; Wessels, 1977, 123–127). In morphogenesis of head structures, a similar pattern is observed, as shown by Hunt *et al.* (1991). Each of the anterior branchial arches is populated by neural crest cells that express a particular, unique set of homeobox genes of the *Hox-2* cluster, determined initially by the region of the neural plate from which they derive; and the overlying ectoderm comes to express the same homeobox genes as the apposed neural crest cells. A *third example* concerns homeobox gene expression in the differentiating kidney, in which various homeobox genes are expressed (e.g. Graham *et al.* 1988; Erselius *et al.* 1990; Kress *et al.* 1990; Vogels *et al.* 1990). *Hox-3.2* is expressed in cortex and also in mesenchymal cells of the developing kidney, for instance, and *Hox-2.3* is expressed in both ducts and tubules in mesonephros and metanephros. Formation of the kidney is regarded as a metameric, anteroposterior process, and the expression of homeobox genes in kidney preserves regionality, in that only *Hox* genes expressed at certain anteroposterior axial positions are also expressed in the developing kidney. Furthermore, expression of the same homeodomain regulators clearly occurs in the various interacting cell types that give rise to the differentiated organ, i.e. duct, mesenchyme, etc. (Holtfreter, 1944; Burns, 1955). An additional striking feature is that the mesenchyme cells that express *Hox-3.2* also express TGFβ1 at the same time (Schmid *et al.* 1991). In fact, many similar parallels appear to exist between organ- and tissue-specific patterns of expression of growth factors (e.g. see Pelton *et al.* 1990; Millan *et al.* 1991; Jones *et al.* 1991), and of retinoic acid receptors and binding proteins (Dollé *et al.* 1990), and the patterns of expression of various homeobox genes, for example in developing heart, lung, stomach, prevertebrae, limb buds, etc. Similarly, in the mouse *Wnt5a*, a mammalian homologue of the *Drosophila wingless* gene, is expressed throughout the posterior region of the late gastrula, and in a graded distribution in the developing limb buds that is independent of cell type or tissue (Gavin *et al.* 1990). Parallels between morphogen distribution and homeodomain proteins or their mRNAs have been noted (e.g. Robert *et al.* 1989; Ruberte *et al.* 1991; Smith and Eichele, 1991; Jones *et al.* 1991) and are likely to become far more obvious when we have higher resolution knowledge of which specific ligands (and their receptors) and which specific homeodomain regulators are present in the key cell populations at the time of formation of each organ system. A *fourth example* is afforded by developing limb buds in *Xenopus* (Oliver *et al.* 1988*a*) and feather buds in chicken (Chuong *et al.* 1990). A homeodomain protein called *XlHbox-1* (recognized by an antibody) is expressed in both morphogenetic processes. Expression in the *Xenopus* limb buds is confined to the forelimbs, and when it first appears it defines the region or 'field' of lateral plate mesoderm where the buds will form. Early in limb bud morphogenesis expression is high anterior–proximal and low posterior–distal; i.e. it is of opposite polarity to a morphogenetic inductive process initiated at the posterior 'zone of polarizing activity' (see Wanek *et al.* 1991; Noji *et al.* 1991; for recent evidence that this inductive morphogenesis could be mediated by short-range cell-to-cell interactions, or by an unknown morphogen, but not by a retinoic acid gradient). Many intercellular interactions clearly must occur within the limb bud mesenchyme, and it is significant that both it and the overlying ectoderm are positive for *XlHbox-1*. In feather bud morphogenesis, the mesoderm provides inductive direction and, through its immediate interaction with adjacent ectoderm, it generates the feather

epithelial placode, and then the feather follicle. In the mesodermal component, the *XlHbox-1* nuclear protein is again graded in distribution, high in the anterior–proximal region of the bud, and all the interacting ectoderm cells *also* express the protein relatively strongly.

It is to be stressed that in many cases homeobox genes are not expressed in *both* inducing and responding tissues, contrary to these examples. In the mouse limb bud, for instance, transcripts of the *Hox-5* gene cluster form an elegantly precise, nested set of spatial zones of expression, centered at the posterior-distal site named the 'zone of polarizing activity'; but these genes are expressed only in the mesodermal component (Dollé *et al.* 1989). Similarly, in the lung, kidney, stomach and trachea, e.g. the paralogous *Hox-1.4*, *Hox-2.6* and *Hox-5.1* genes are expressed in mesoderm but not in endodermal endothelial layers (Gaunt *et al.* 1989); in *Xenopus*, the *XlHbox-8* gene is expressed just before, and during differentiation of a region of the endoderm that includes the presumptive duodenum, the pancreas and its duct system, i.e. in a number of *different* local endodermal cell types (Wright *et al.* 1988). Different homeobox genes are expressed in the overlying gut mesoderm (Oliver *et al.* 1988*b*). Supposing that the downstream targets of homeodomain regulators are indeed genes encoding ligands and receptors, there are many reasons why only one of the interacting cell types might be observed to be expressing a given homeobox gene: the ligand might be regulated by one homeodomain protein and the receptor by another, as in Fig. 2B, right box (see legend); the receptor, or the ligand, could be very broadly expressed and thus only the other would *require* spatial regulation; the cell 'type' to which expression is observed to be confined, e.g. an organogenic mesenchyme, might in fact be heterogeneous, and the cells may be engaged in interactions with one another; or these cells might be required to interact with another cell type not noticed at the time of the observation. For example, as the above references show, many homeobox genes that are expressed in the mesoderm of given organs are also expressed in neural crest, derivatives of which ultimately contact and innervate these organs.

Phenotypic consequences of perturbing regional homeobox gene expression

When expression of a homeodomain regulator is prevented in an amphibian embryo, or ectopic expression is forced, there are morphological consequences of the sort one might imagine were a local morphogenetic inductive program interrupted by failure of, or ectopic, ligand–receptor presentation. In general the effects are confined to those regions of the body plan to which expression is normally confined, indicating that the normal downstream function of homeodomain regulators is not mediated by long-range diffusible morphogens. Several informative cases have been reported from *Xenopus*. *XlHbox-1* is expressed in two transcripts, 'long' and 'short', which may function antagonistically. The short protein is expressed more anteriorly, in CNS, neural crest, mesoderm and internal organs (Oliver *et al.* 1988*b*). Wright *et al.* (1989) showed that injection into the fertilized egg of an antibody recognizing the more posterior 'long' protein causes the upper spinal cord to begin development as a hindbrain, normally the next most anterior CNS structure, while a similar effect is produced by injection of excess 'short' protein mRNA. This result suggests modular, local control of morphogenetic fate, involving reorganization of similar cell types; thus motor nerves are generated by both normal and transformed CNS elements but they are organized differently. Synthetic transcripts of a homeobox gene called *Xhox-1A*, that is normally expressed in axial structures of the trunk, were injected into early blastomeres by Harvey and Melton (1988), with the primary result that somitic morphogenesis was disorganized. Nonetheless, molecular muscle-specific markers were expressed as in normal somites, emphasizing that excess *Xhox-1A* expression affects *tissue organization*, i.e. intercellular arrangement and communication, rather than differentiation *per se*. The effects of ectopic expression of *Xhox-3*, a homeobox gene normally expressed in a declining posterior-to-anterior gradient, were reported by Ruiz i Altaba and Melton (1989*a*). Injection of this transcript into early anterior blastomeres permits normal gastrulation, and the formation of differentiated notochord, muscle and neuronal cell types, but it then results essentially in the deletion of head formation. An inductive process appears to have been affected, since the anterior neural plate is normally specified inductively by underlying anterior mesoderm and, though the mesodermal cells and the neural plate are in place in the experimental animals, they evidently fail to interact properly. The effects of ectopic expression of homeodomain regulators during development have also been reported in the mouse (reviewed by Kessel and Gruss, 1990). In one interesting case, overexpression of a germline *Hox-1.4* construct under the control of an SV40 promoter resulted in apparent failure of innervation of the large intestine (Wolgemuth *et al.* 1989) either because of an effect on the innervating neural crest derivatives, or on the gut mesenchyme during development. In either case, the result could again be interpreted as interference with specific processes of intercellular interaction. Germline deletion of the *Hox-1.5* gene (Chisaka and Capecchi, 1991) in the mouse causes a series of defects in structures that derive from the pharyngeal arches and pouches, including thymus, parathyroid, thyroid, arteries and lower facial structures. These defects can be interpreted as the direct downstream consequences of failures of inductive interactions between cephalic neural crest and pharyngeal endoderm, mesoderm and ectoderm (Chisaka and Capecchi, 1991). Thus the *Hox-1.5* gene might be specifically required for the regional manifestation of the ligands and receptors, signalling machinery, etc. that modulate these interactions. Another striking and relevant observation is that, as shown by Thomas and Capecchi (1990) and McMahon and Bradley (1990), germline

disruption of the *Wnt-1* gene results in what appear to be *modular regional* defects in the ongoing inductive processes by which morphogenesis of the cerebellum and mesencephalon occurs. The mouse *Wnt-1* gene apparently encodes a secreted ligand homologous to that produced by the *wingless* gene of *Drosophila* (van den Heuvel *et al.* 1989), and it is a reasonable speculation that there is a relation between *Wnt-1* expression, and the expression of at least one homeodomain regulator, *engrailed*, which may be required for this aspect of CNS development (McMahon and Bradley, 1990).

The arguments in this section suggest a mechanism by which homeodomain regulators may work in the late embryonic and postembryonic vertebrate development. Using this idea as a lens, it is interesting now to examine again the three major types of early embryonic process, and their evolutionary interrelationships.

Embryonic and postembryonic development in Metazoa

Different modes of activation of regionally expressed homeobox genes in early development

The *later* developmental processes by which organs are formed and by which morphogenesis of three-dimensional multicellular structures occurs are probably common to all animal forms above sponges. Assuming the downstream functions of regionally expressed homeobox genes envisioned in respect to vertebrate development, we might therefore expect to encounter these same functions universally, wherever short-range inductive morphogenetic functions are required. This should, for example, include the regional inductive interactions that mediate anteroposterior diversification in bilateral animals. On the other hand, the *initial mechanisms by which an egg turns into an embryo* capable of later generating subsequent regional morphogenesis, are clearly different. If regionally expressed homeobox genes control local inductive morphogenesis, then in the types of embryo that we have discussed the mechanistic meaning of these differences will be revealed, in part, by the ways that homeobox genes are initially activated.

In Type 1 embryos cell specification occurs in a fixed spatial geometry with respect to lineage, by conditional and autonomous processes. There is no obvious requirement for early *regional* homeobox gene expression (i.e. expression in cells that will give rise to a variety of different cell types), since the positions of the initial embryonic tissues with respect to the larval body plan are already established by lineage, cleavage pattern and the geometry of gastrular invagination. Of course such genes might indeed control conditional specification by intercellular interaction in particular lineages, or be used for other functions in early Type 1 development. The arguments given above would suggest that *regional* control functions will only be required in *postembryonic* Type 1 development, as lineage relations blur, cells multiply and migrate, and are organized *de novo* into organs and structures interrelated in position (except perhaps in heterochronic direct developing species). Unfortunately, not much is known about spatial patterns of homeobox gene expression in Type 1 embryos. A homeobox gene called *AHox-1* is expressed in ascidian embryos but not until larval and juvenile stages (Saiga *et al.* 1991). Transcripts appear in gut, at the time of the functional differentiation of this organ, and in juvenile coelomocytes. The ascidian endoderm derives from a fixed embryonic lineage, as described earlier, and juvenile coelomocytes are probably also of endodermal lineage (Whittaker, 1990). In the sea urchin embryo, transcripts of a gene called *TgHbox-1* accumulate in a particular region of the aboral ectoderm, in mid–late embryogenesis (Angerer *et al.* 1989). However, cytotypic marker gene expression in aboral ectoderm is initiated in cleavage, long prior to *TgHbox-1* expression, so the conditional process of initial aboral ectoderm specification cannot depend on the activity of this homeobox gene. Expression in the late embryo appears to be confined to a single clonal lineage, which is the descendant of the VA founder cell, located at the posterior vertex of the larva. The postembryonic role of this regulator could be to mediate intercellular interactions in larval skeletogenesis, which will be active in the vertex region. In *C. elegans* a homeobox gene called *mab-5* is required for postembryonic morphogenesis in the posterior region, where it is expressed after completion of embryonic founder cell specification in many different cell types (Kenyon, 1986; Costa *et al.* 1988). Among a variety of morphogenetic functions *mab-5* is required for development of certain male sensory structures, and in this process *mab-5* expression is under the control of a cell signalling system (Waring and Kenyon, 1990, 1991). Thus this gene both responds to, and probably regulates (to account for its morphogenetic effects), specific intercellular interactions, just as is typical for regionally expressed homeobox genes in postembryonic vertebrate development.

In the Type 2 embryos of vertebrates the initial regional expression of homeobox genes is apparently activated by long-range diffusible morphogens that are responsible for the broad organization of the body plan. Regional expression of specific homeobox genes has been related to the activity of known morphogens in *Xenopus*. Ruiz i Altaba and Melton (1989*b*) showed, for example, that *Xhox-3* is transcribed in isolated animal caps if these are exposed to growth factors of the TGFβ and bFGF families that are known to be required for the initial axial specification of mesoderm. As we recall *Xhox-3* transcripts are normally concentrated in the dorsal posterior mesoderm at late gastrula–neurula stages, and the accumulation of these transcripts is stimulated by high levels of bFGF, a known morphogen for posterior-dorsal mesoderm. High levels of a TGFβ relative (XTC-MIF), that induce anterior-dorsal mesoderm, correspondingly depress *Xhox-3* expression. Similarly Cho and DeRobertis (1990) showed that bFGF activates *XlHbox-6*, which is expressed in the posterior CNS (Sharpe *et al.* 1987; Wright *et al.* 1990),

while XTC-MIF also activates *XlHbox-1*, which is expressed in the anterior trunk (see above). The causal linkage between *early* embryonic processes that initially specify anteroposterior fates and *later* regional homeobox gene expression is further demonstrated by the effects of UV treatment of zygotes, and Li^+ treatment of early embryos (Ruiz i Altaba and Melton, 1989*c*). Ectopic respecification of anterior-dorsal fates toward posterior-ventral fates by UV irradiation results in elevation of *Xhox-3* expression, and the ectopic respecification toward anterior-dorsal fates caused by exposure to Li^+ results in depression of *Xhox-3* expression. Clearly *Xhox-3* is not autonomously specified with respect to its domain of expression. Another diffusible morphogen that effects anteroposterior specification in the CNS and mesoderm of *Xenopus* is retinoic acid (Durston *et al.* 1989; Sive *et al.* 1990). This agent promotes posterior specification, and it also promotes expression of *XlHbox-6*. Like the transcripts of the homeobox genes whose activity they initially control, in gastrula–neurula stage embryos, the effects of retinoic acid are distributed regionally, in that developmental sensitivity to this morphogen is observed at the organ or body plan level. Thus retinoic acid treatment alters development of anterior CNS, cement gland and other anterior structures that include contributions from *both* ectodermal and mesodermal derivatives (Sive *et al.* 1990). These early effects of retinoic acid in *Xenopus* embryos can at least in part be accounted for as modulation of the anterior mesoderm specification initially induced by growth factors (Ruiz i Altaba and Jessell, 1991). Thus retinoic acid apparently acts downstream of *Xhox-3* expression in the mesoderm but upstream of other homeobox gene expressions involved in regional morphogenesis. Retinoic acid probably also exercises important developmental functions in mammalian and avian embryos. Thus it activates homeobox genes in cultured EC cells (e.g. Deschamps *et al.* 1987; Simeone *et al.* 1990). Forced ectopic expression of the *Hox-1.1* gene in transgenic mice causes a distinct subset of the morphological phenotypes caused by high doses of retinoic acid, and that is probably due to failure of morphogenetic neural crest cell functions in the head and face (Balling *et al.* 1989). This interpretation implies that in later development as well, the morphogenetic effects of retinoic acid may be directly exercised through homeobox gene activation.

In Type 3 embryonic process homeobox genes are initially activated in an entirely different way, dependent neither on diffusible growth factors, nor on short-range cell interactions or cell lineage. As discussed above, in these embryos homeobox genes are used to generate the embryonic body plan *ab initio*, rather than being used to carry morphogenesis onward *following* prior generation of the body plan by other means. The properties of pair-rule homeobox gene expression that enable them to regulate one another are utilized to set up precellular spatial patterns of regulatory gene expression. Among these properties are regulatory domains that include positively and negatively acting target sites for the homeodomain protein products of other such genes, as well as for other transcription factors (e.g. see Dearolf *et al.* 1989; Biggin and Tjian, 1988; Hayashi and Scott, 1990); and also their clustered genomic organization, as discussed in an interesting way by Peifer *et al.* (1987). As stressed earlier, homeobox gene interactions are only one of several unique regulatory devices utilized in precellular *Drosophila* embryos for spatial patterning, but their mode of initial utilization highlights the distinction between Type 3 and other forms of early embryonic processes. After the initial spatial patterning is established, however, i.e. from the beginning of gastrulation on, *Drosophila* embryos utilize their homeobox genes in ways more similar to those seen in postembryonic vertebrate development. As noted above, homeodomain regulators of the segment polarity group such as *engrailed*, evidently control expression of ligands and receptors (directly or indirectly). A very interesting example recently described is the relation between *dpp* and *wingless* expression, and homeotic gene expression, in the midgut of *Drosophila* (Panganiban *et al.* 1990; Immerglück *et al.* 1990; Reuter *et al.* 1990; Reuter and Scott, 1990). The products of the *dpp* gene, a TGFβ family member, and of the *wg* gene, apparently a diffusible ligand, are required for the inductive morphogenesis of the midgut. These factors are produced in the visceral mesoderm, and the genes encoding them are evidently downstream targets of homeotic genes that regulate midgut development. In parasegment8 expression of the *AbdA* gene causes *wg* expression and blocks *dpp* expression, and, in parasegment 7, *Ubx* gene expression causes *dpp* expression. The effects observed in various ectopic expression experiments in which these homeotic selector genes were driven by heat-shock promoters, suggest that the *dpp* gene may be a direct target of the homeodomain regulatory factors (Reuter *et al.* 1990). The *dpp* factor in turn affects *Ubx* expression in the visceral mesoderm, and induces *labial* gene expression in the adjacent endodermal epithelium. Mutations that prevent *dpp* expression in the midgut have *modular* effects on midgut morphogenesis, here failure of given local structures to be formed. In the midgut the exclusive regional patterns of homeobox gene expression are thus mediated by intercellular inductive effects on homeobox gene expression, by the negative interactions of homeodomain regulators with other homeobox genes within the same nucleus, and by the activation of intercellular signalling systems by homeodomain regulators that in turn effect induction of homeobox genes (Tremml and Bienz, 1989; Reuter *et al.* 1990). Similar sets of regulatory interactions are likely to be involved in control of homeotic gene function in other regions of the post-cellularization *Drosophila* embryo, and may be used to define the boundaries of the multicellular regions of homeodomain regulator expression in all forms of embryo. That is, intercellular signalling that regulates homeobox gene expression is likely to be required at the lateral boundaries of regions that are defined by the patterns of expression of these genes.

The *modular nature of the downstream effects* of homeodomain regulators depends of course on their regional expression. This suggests that true *homeotic* effects, i.e. exchange of one modular morphogenetic program for another, might operate by simply causing a switch of one set of downstream ligand–receptor interactions that promote a certain morphogenesis for a different one.

Evolutionary derivation of embryonic processes

The implication of Fig. 1 is that Type 2 and 3 embryogenesis are, in evolutionary terms, derived. The view adduced here is that, while all Metazoa probably utilize the same kinds of intercellular, conditional specification processes for organogenesis and morphogenesis, there are several quite different processes by which gastrula-stage embryos are generated from eggs. Such differences can be seen starting to occur in direct developing forms of various Type 1 taxa, and are extensively amplified in what I have called Type 2 and Type 3 embryonic processes. One way of imagining how this might have come about is to regard both of these as heterochronic derivatives of Type 1 processes. Type 3 embryos exemplify a revolutionary, early use of regional homeobox gene expression, together with other special regulatory adaptations. Furthermore, in these embryos the autonomous, precellular development of spatial regulatory gene expression patterns is, until the end of this stage of the process, separated from direct control of downstream target genes that mediate intercell interaction. In Type 2 embryonic process, regional homeodomain regulators are used extensively in mid–late embryonic development, because even at these relatively early stages these embryos utilize the same kinds of morphogenetic mechanisms that continue in postembryonic development. It is a fascinating thought that the progenitors of the advanced insects, and the different progenitors of the vertebrates, both found in the available library of postembryonic genetic regulatory devices ways to replace the lineage-based processes of early embryogenesis, which from the beginning of the Cambrian to the present probably provided the universal basic pathways for embryogenesis.

Needless to say, I have relied heavily on the thoughtful critical advice, and the expert insights of many colleagues who generously expended their valuable time in reviewing drafts of this manuscript. I cannot too strongly express my appreciation to the following people: Frank Calzone, UC Irvine; Rick Firtel, UC San Diego; Gary Freeman, University of Texas; Antonio Garcia-Bellido, Centro de Biologia Molecular, Madrid; Mike Levine, UC San Diego; Andrew McMahon, Roche Institute; Jim Posakony, UC San Diego; Richard Whittaker, Marine Biological Laboratory; and Roy Britten, Andy Cameron, Scott Fraser, Barbara Hough-Evans, Howard Lipshitz, Ellen Rothenberg, and Paul Sternberg, all of Caltech. This work was partially supported by a grant from the National Institutes of Health (HD-05753).

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(Accepted 17 June 1991)